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Optimization of a two-plasmid system for the identification of promoters recognized by RNA polymerase containing *Staphylococcus aureus* alternative sigma factor σ^B

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Abstract

We optimized a previously established two-plasmid system for the identification of *Staphylococcus aureus* promoters that are recognized by the alternative transcription factor σ^B. The method allowed the identification of 18 *S. aureus* σ^B-dependent promoters, 12 of which are reported here for the first time to be σ^B-dependent. S1-nuclease mapping of the respective transcriptional start points revealed that all the promoters contained sequences exhibiting high similarity to the consensus sequence of *Bacillus subtilis* σ^B-dependent promoters. The promoters governed expression of genes encoding proteins proposed to be involved in various cellular functions, including the stress response genes and virulence-associated *clfA* gene for fibrinogen-binding clumping factor. Comparison of the nucleotide sequences upstream of the identified transcription start points identified a σ^B consensus promoter (GttTaa-N_{12-15}-gGGTAt) that is highly homologous to that of σ^B of *B. subtilis*.

Keywords: Bacterial virulence; Pathogenesis; Promoter; RNA polymerase; Sigma factor; *Staphylococcus aureus*; Stress response

1. Introduction

In their natural habitats, bacteria are exposed to various stresses that elicit the production of so-called general stress response proteins, having non-specific, essential, and protective functions under stress. The response to these stresses is often mediated by alternative sigma factors of RNA polymerase. In the Gram-positive *Bacillus subtilis*, the general stress response is mainly governed by the alternative transcription factor σ^B. Several approaches identified the *B. subtilis* σ^B regulon to comprise more than 200 genes [1,2]. Alternative sigma factors homologous to σ^B have also been found in other Gram-positive bacteria, including pathogenic *Listeria monocytogenes* [3] and *Staphylococcus aureus* [4,5].

The genetic organization of the *Staphylococcus aureus sigB* operon resembles in part that of its counterpart in *B. subtilis* [4,5]. Similarly, the *S. aureus* σ^B has been shown to be involved in general stress response [6]. A proteomic approach analyzing cytoplasmic protein fractions identified 27 proteins (Csb) to be positively activated by σ^B in *S. aureus* [7], suggesting the *S. aureus* σ^B regulon to comprise a much higher number of genes, which might be as large as that of *B. subtilis*. Aiming at the identification of sigma factor-dependent promoters, we previously developed a method based on two *Escherichia coli* compatible plasmids. A particular sigma factor heterologously expressed from one plasmid can interact with the *E. coli* RNA polymerase core enzyme and the resulting holoenzyme recognizes a promoter present in a library of DNA fragments cloned in the second compatible plasmid, upstream of a promoterless *lacZ*α reporter gene [8]. In the present work, we adapted this method for the identification of promoters recognized by *S. aureus* σ^B.

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2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

S. aureus COL strain [9] was used for chromosomal DNA preparation and polymerase chain reaction (PCR) amplifications. The *E. coli* promoter probe plasmid pSB40N and the expression plasmid pAC7 are described in [8]. *E. coli* XL1 Blue (Stratagene) was used as a host for cloning experiments. Conditions for *E. coli* growth and transformation were as described in [10]. For RNA isolation, *E. coli* with the corresponding plasmids was inoculated in LB medium [10] supplemented with ampicillin (50 μg ml^{-1}) and chloramphenicol (40 μg ml^{-1}), grown at 37°C to exponential phase ($OD_{600} = 0.3$), and expression of *S. aureus sigB* was induced for 3 h with 0.0002% arabinose.

2.2. DNA manipulations

DNA manipulations in *E. coli* were performed as described in [10]. Nucleotide sequencing was performed by the chemical method [11]. Plasmid pAC7-sigB containing the *S. aureus sigB* gene under the control of the tightly regulated arabinose-inducible P_{BAD} promoter [12] was constructed as follows: the *sigB*-containing 770-bp DNA fragment was PCR-amplified from *S. aureus* COL chromosomal DNA using an upstream primer (5′-GATCATATGGCGAAAGAGTCGAAATCAGC-3′) and a downstream primer (5′-GCGAAGCTTCAAATTCTATTGATGTGCTGC-3′), which introduced an *Nde*I site to the translation initiation codon and a *Hin*dIII site downstream of the stop codon. The PCR fragment was digested with *Nde*I and *Hin*dIII, ligated into pAC7, cut with the same enzymes, and subsequently transformed into *E. coli* XL1 Blue, resulting in pAC7-sigB. The nucleotide sequence of the cloned *sigB* gene in pAC7-sigB was confirmed by sequencing, and was identical with the sequence under GenBank accession number Y09929. The well-characterized *S. aureus* σ^B-dependent *asp23p* promoter [13] was cloned in the promoter probe plasmid pSB40N as follows: the *asp23p*-containing 580-bp DNA fragment was PCR-amplified from *S. aureus* COL chromosomal DNA using an upstream primer (5′-CGGGGATCCTGGATTATACAAAGACTTCG-3′) containing a *Bam*HI site and a downstream primer (5′-CGGCTCGAGTTGATGAATTAACTCCATCG-3′) containing an *Xho*I site. After digestion with *Bam*HI and *Xho*I, the PCR fragment was ligated into pSB40N cut with the same enzymes, and transformed into *E. coli* XL1 Blue, resulting in pSB40N-asp23P1. The nucleotide sequence of the cloned fragment was confirmed by sequencing, and was identical with the corresponding sequence under GenBank accession number AP0031336.

An *S. aureus* COL genomic library was prepared by cloning 0.5–1.2-kb partial *Taq*I chromosomal DNA fragments into the *Cla*I site of pSB40N. About 160 000 original clones arisen from transformation of *E. coli* XL1 Blue were used for plasmid isolation with the Qiagen plasmid purification kit (Qiagen, Hilden, Germany). The clones were statistically checked for the presence of insert, and all the picked up clones contained fragments in the range of 0.5–1.2 kb.

2.3. Detection of E. coli clones containing the S. aureus σ^B-dependent promoter fragment

The plasmid pSB40N-asp23P1 was transformed in parallel into *E. coli* XL1 Blue containing pAC7 and pAC7-sigB, and the clones were selected on LBACX-ARA plates (LB medium with 5 g l^{-1} lactose, 100 μg ml^{-1} ampicillin, 40 μg ml^{-1} chloramphenicol, 20 μg ml^{-1} X-gal, and 2 μg ml^{-1} arabinose). The colonies were screened after 24 h growth at 37°C. Likewise, the *S. aureus* COL genomic library was transformed into *E. coli* XL1 Blue containing the compatible plasmid pAC7-sigB, and clones selected on LBACX-ARA plates. Blue clones were inoculated into 1 ml LB+Ap (100 μg ml^{-1}) liquid medium and grown overnight at 37°C. Cells were pelleted, suspended in 200 μl STE buffer (0.1 M NaCl, 10 mM Tris–HCl pH 8, 1 mM EDTA) with 0.5 mg ml^{-1} lysozyme, incubated 5 min at room temperature, boiled 1.5 min, and centrifuged for 10 min at 13 000 rpm. 1 μl of supernatant was transformed in parallel into *E. coli* XL1 Blue strains harboring either pAC7-sigB or pAC7, and plated on LBACX-ARA.

2.4. Isolation of RNA and S1-nuclease mapping

Isolation of total RNA and high-resolution S1-nuclease mapping were performed as previously described [14]. Samples (40 μg) of RNA (estimated spectrophotometrically) were hybridized to approximately 0.02 pmol of a suitable DNA probe labelled at the 5′ end with [γ-^{32}P]ATP (approximately 3×10^6 cpm $pmol^{-1}$ of probe). The probes used were prepared by PCR amplification from the corresponding pSA plasmids using the 5′ end-labelled universal oligonucleotide primer −47 (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) from the *lacZ*α coding region, and the primer mut80 (5′-GGGTTCCGCGCACATTTCCCCG-3′) from the 5′ region flanking the polylinker of pSB40N. The protected DNA fragments were analyzed on DNA sequencing gels together with G+A and T+C sequencing ladders derived from the end-labelled fragments [11].

3. Results and discussion

3.1. Investigation of the two-plasmid system to identify S. aureus σ^B-dependent promoters

In order to identify new *S. aureus* σ^B-dependent pro-

moters, we tried to optimize a previously developed method for the identification of promoters recognized by a particular σ factor of RNA polymerase [8]. The *S. aureus sigB* gene was PCR-amplified, and the resulting *sigB*-containing DNA fragment was cloned into the plasmid pAC7 [8] under the control of a tightly regulated arabinose-inducible P_{BAD} promoter [12], resulting in pAC7-sigB (Section 2). To test the system, the well-characterized *S. aureus* σ^B-dependent *asp23p* promoter [13] was cloned in the compatible promoter probe plasmid pSB40N [8], upstream of the *lacZα* reporter gene. The resulting plasmid pSB40N-asp23P1 was transformed in parallel into *E. coli* XL1 Blue containing either pAC7 or pAC7-sigB, and transformants were selected on LBACX-ARA plates. Both negative (containing pAC7) and positive (containing pAC7-sigB) clones grew comparably. Transformants containing pAC7 produced uncolored colonies, indicating that the *S. aureus asp23p1* promoter is not recognized by any form of *E. coli* RNA polymerase holoenzyme. However, transformants containing pAC7-sigB were blue on the selective LBACX-ARA plates, indicating that the activity of the *asp23p1* promoter is dependent upon arabinose-induced heterologous expression of the *S. aureus sigB* in *E. coli*. Moreover, transcription start point (TSP) analysis of the *asp23p1* promoter in the *E. coli* two-plasmid system after arabinose-induced expression of *S. aureus sigB* identified a TSP (Fig. 1) that was close (three nucleotides upstream) to the one published previously [13]. Thus, the *S. aureus* σ^B-dependent *asp23p* promoter is similarly recognized by the heterologous RNA polymerase holoenzyme containing *S. aureus* σ^B, as by its homologous holoenzyme in *S. aureus*. This is consistent with the recently published structures of two alternative sigma factors that are essentially identical in two critical domains, σ_2 and σ_4, for binding of core RNA polymerase and promoter regions with primary sigma factors [15,16].

Based on these results, we have concluded that the two-plasmid system could be used for identification of new *S. aureus* σ^B-dependent promoters. For this purpose, an *S. aureus* COL library, cloned into pSB40N (Section 2), was used to transform *E. coli* XL1 Blue containing pAC7-sigB. After screening of about 160 000 colonies on LBACX-ARA plates, 1419 blue clones that represented promoters active in *E. coli* (including σ^B-dependent promoters) were picked up. To identify the σ^B-dependent promoters, plasmid DNA from the clones was transformed in parallel into *E. coli* with pAC7 and pAC7-sigB, and colonies were screened on LBACX-ARA plates. Clones containing plasmids with σ^B-dependent promoters were blue in *E. coli* XL1 Blue with pAC7-sigB and white in *E. coli* XL1 Blue containing pAC7. Clones with σ^B-independent promoters were blue in both strains. Using this screen we identified 53 positive clones containing σ^B-dependent promoters (plasmids pSA1–pSA53). Sequencing of the DNA fragments revealed 18 representatives.

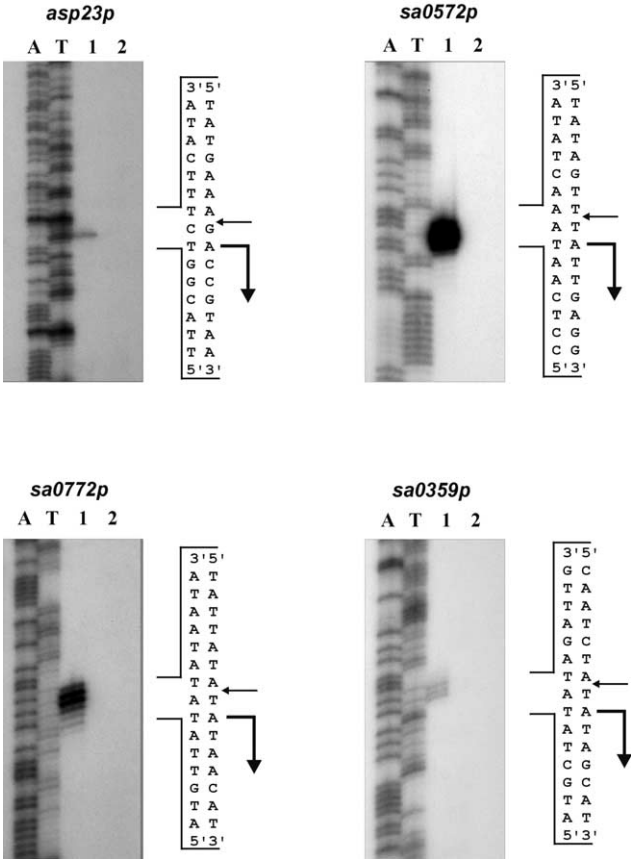

Fig. 1. Examples of TSP determinations of the *S. aureus* σ^B-dependent promoters by high-resolution S1-nuclease mapping in the *E. coli* two-plasmid system. The 5′-labelled DNA fragment (0.02 pmol) was hybridized with 40 μg RNA and treated with 100 U of S1-nuclease as described in [14]. RNA was isolated from exponentially growing *E. coli* containing the corresponding pSA plasmid (bearing a DNA fragment with the σ^B-dependent promoter indicated) and pAC7-sigB (lane 1) or pAC7 (lane 2), induced for 3 h with arabinose. The RNA-protected DNA fragments were analyzed on DNA sequencing gels together with G+A (lane A) and T+C (lane T) sequencing ladders derived from the end-labelled fragments [11]. Thin horizontal arrows indicate the positions of RNA-protected fragments and thick bent vertical arrows indicate the nucleotide corresponding to TSP. Before assigning the TSP, 1.5 nucleotides were subtracted from the length of the protected fragment to account for the difference in the 3′ ends resulting from S1-nuclease digestion and the chemical sequencing reactions.

3.2. *Characterization of the* S. aureus σ^B*-dependent promoters*

In order to identify TSPs of the 18 identified σ^B-dependent promoters, high-resolution S1-nuclease mapping was performed using RNA isolated from *E. coli* with a particular pSA plasmid and pAC7-sigB grown to exponential phase and induced with arabinose and 5′-labelled DNA probes for a particular promoter (Section 2). The experiments are documented in Fig. 1. No RNA-protected fragments were identified with a control RNA from *E. coli* containing particular pSA plasmid and pAC7. However, RNA-protected fragments were identified with all the positive samples (RNA from *E. coli* containing corresponding

		−35		−10	
sa1984p	(*asp23p*) AGTACTTATCATCGTTTTAT	**GTTTAA**	TTGGTGTAGGTATT	**GGGTAT**	ATGAAAG**A**
sa1987p	(*opuDp*) GTCTATATAAAATTACAAAT	**GATTAA**	AATGTTGCAGTTAT	**GGGTAT**	GAACTTACC**A**
sa2336p	(*clpLp*) TAAAGTTTTAAAAATATTCT	**GTTTTA**	TCACCTATTATTAG	**TGGAAA**	AGTACAATT**G**
sa0742p	(*clfAp*) TGTATAATTCCATTAACAGA	**GATTAA**	ATATATCTTTAAA	**GGGTAT**	ATAGTTA**A**
sa1452p	(*csb8p*) TAAAAACTTCATATTATAAT	**GTTTAG**	CGAACCTCCTTAG	**TGGTAT**	ATAAATAT**A**
sa1946p	(*csb9p*) GATTTTTATAGTTGTAACAA	**GTTTTA**	CATATCTCATAAAG	**TGGTAT**	GGCATAGAGA**A**
sa0455p	GTTATGAATTTAATGAATGA	**GTTTAA**	AGCCCATGTAAAAG	**GGGTAT**	CAGTACTT**G**
sa0572p	ATTGTTTATAAAATGGAAGC	**GTATAT**	AGAATGAAGGTT	**GGGTAT**	ATAGTTT**A**
sa1143p	AATTTGGGAAGGTAAATCAG	**GTTTAT**	TAACTATTGCAGG	**TGGTAA**	ATTAACAG**G**
sa0772p	AAAAATTATTTAAGTAAAAT	**GTTTAG**	ATAATTTTTCAGT	**GGGTAA**	GTATTATAT**A**
sa2298p	TAAAAAATTGTGTTTAATAT	**GTTTCA**	TTTTATAATTATGG	**TGGTAT**	ATAACATGA**A**
sa0752p	CATAAGTAATGATGATTTTA	**GTTTAA**	AAGATAATGTGACG	**GGGTAA**	AACGCAAT**G**
sa2451p	TACACATACGTTACATAATT	**GATTCA**	TTTTTATAGAAAC	**GGGTAA**	AAATGATAA**A**
sa0633p	TTATAGATGAAAGGTACAGC	**GTTTTA**	AACCTTATTTTAA	**GGGTAT**	GTATTAATT**A**
sa2309p	TGTCATGCACCTTTTACTTC	**GTTTAA**	GTTAAAATAGAATA	**GGGAAA**	TATTATA**A**
sa2219p	TATAAGGATGAGAGGAAAGA	**GACTAA**	ATTTGCTGTGAAA	**GGGTAT**	AAAGGTTGAA**A**
sa0359p	AAAATATTTGTAATAATTAT	**GAATAA**	AATTAAAAACAAG	**GGGTAA**	TACAATCTAT**A**
Mw0922p	AATTAAATTGTGACAATGAC	**GTTTAA**	AAAGCATATATGAAT	**GGGTAT**	ATAGTTCGT**A**
B. subtilis σ^B consensus		**GTTTAA**	**N_{12-14}**	**GGGTAT**	
S. aureus σ^B consensus		**GttTaa**	**N_{12-15}**	**gGGTAt**	

Fig. 2. Nucleotide sequence alignment of the identified *S. aureus* σ^B-dependent promoters. The corresponding −10 and −35 regions are depicted in bold. The TSP is in bold and underlined. The consensus sequences of *B. subtilis* σ^B-dependent promoters [17] and *S. aureus* σ^B-dependent promoters are below the alignment.

pSA plasmid and pAC7-sigB) (Fig. 1). In all cases, positions of the RNA-protected fragments located a TSP downstream of a sequence highly similar to that of the consensus sequence of *B. subtilis* σ^B-dependent promoters [17] (Fig. 2). This is not surprising, as both sigma factors are very similar in the regions 2.4 and 4.2, which are supposed to be of importance for the recognition of the −10 and −35 promoter regions, respectively [4,5]. Therefore, it is likely that both sigma factors might recognize similar promoters.

3.3. Characterization of the S. aureus σ^B-dependent genes

Comparison of the sequences of the identified *S. aureus* σ^B-dependent promoters with the published genomic sequence of *S. aureus* N315 (accession number NC_002745) allowed the identification of the open reading frames that are governed by the promoters. The *S. aureus* genes directed by the identified σ^B-dependent promoters and their chromosomal location in *S. aureus* N315 chromosome are described in Table 1. All promoters were located upstream of a convergent gene. Among the 18 identified *S. aureus* σ^B-dependent promoters, three promoters, including the well-defined *asp23p* [13], were located within the coding region of an upstream convergent gene (Table 1).

The σ^B regulon previously defined for *B. subtilis* was shown to encode a variety of protein functions involved in metabolic pathways, transport, and other fundamental cellular functions. Many of the genes have been shown to encode proteins directly associated with stress resistance [1,2,17]. Similar to its *B. subtilis* counterpart, *S. aureus* σ^B has been shown to be involved in the general stress response [6]. Moreover, expression of some virulence factors was shown to be altered by σ^B, suggesting the sigma factor to influence *S. aureus* pathogenesis, although no direct effect of σ^B on pathogenicity has been demonstrated yet [6,18,19]. Thus, the main role of the *S. aureus* σ^B regulon might include the general stress response, and likely some virulence functions, as suggested previously [7]. The inferred functions of some of the identified members of σ^B regulon fell broadly in this category. Alkaline shock protein Asp23 has been characterized previously as σ^B-dependent, although no function has been associated with this protein yet [13].

Three further σ^B-dependent genes (*csb8*, *csb9*, and *clpL*) have been previously identified to belong to the *S. aureus* σ^B regulon by a proteomic approach [7]. However, the functions of Csb8 and Csb9 are currently unknown as well [7]. The third gene previously identified by the proteomic approach is the ATP-dependent Clp proteinase chain ClpL, one of the ATPase regulatory subunits of Clp protease. Clp-mediated proteolysis plays an important role in the general turnover of damaged proteins and in regulated degradation of short-lived regulatory proteins. It was also demonstrated to be important for survival during various stress conditions and in virulence [20]. Clp protease consists of a ClpP protease subunit and an ATPase regulatory subunit from the Hsp100 family of chaperones, which determine the substrate specificity on the Clp complex [20]. Two candidates of this family, ClpP and ClpX, have been found to play a critical role in stress response and virulence in *S. aureus* [21], suggesting that ClpL might have a similar function.

The other candidate of σ^B-dependent genes that are likely to be involved in the general stress response is *opuD*. This gene, encoding glycine betaine transporter, was shown to have a role in osmoprotection in *B. subtilis* [22], and it has been shown to be dependent upon σ^B in *L. monocytogenes* [23] and *B. subtilis* [1,2]. Its σ^B dependence in *S. aureus* has been independently documented as well [24], indicating that the σ^B regulon might contribute to osmoprotection in *S. aureus* too. Interestingly, osmoprotective systems have been shown to function as important

virulence factors for certain pathogenic bacteria, including *S. aureus* [25].

Another gene with a known function that has already been suggested to be influenced by σ^B is *clfA*, encoding fibrinogen-binding clumping factor A [18]. ClfA belongs to *S. aureus* surface proteins that allow the pathogen to bind host extracellular matrix proteins. ClfA, together with other fibronectin-binding proteins, has been shown to affect pathogenicity of *S. aureus* [26]. The σ^B dependence of *clfA* expression suggested a role of *S. aureus* σ^B regulon in virulence.

All other members of the *S. aureus* σ^B regulon identified here encode hypothetical proteins with yet unknown functions, or proteins having a similarity to some families of proteins (Table 1). Interestingly, SA0772, containing the domain COG3237 of yet unknown function that is highly conserved among bacteria, is highly similar (79% amino acid identity and 88% similarity) to the previously identified σ^B-dependent protein Csb8 (SA1452) in *S. aureus* [7]. As both proteins are likely to be similar in size (64 and 60 amino acids, respectively), they may constitute a family of paralogous proteins in *S. aureus*. In contrast, their σ^B-dependent homologue in *B. subtilis* (CsbD, YwmG) [1,2] is present only in single copy and has no similarity to any of the other *B. subtilis* genes.

SA2219 is a predicted membrane protein containing the conserved domain pfam04138 of the GtrA family. The members of this family are involved in the synthesis of cell surface polysaccharides in bacteria. This family also includes the cell wall teichoic acid glycosylation protein GtcA of *L. monocytogenes* [27], which shares 25% amino acid identity and 52% similarity with SA2219. Although GtcA, as well as its sequential homologue YwcD in *B. subtilis*, has not been identified to belong to the σ^B regulons of these strains [1,2,23], one of the glucosyltransferases involved in the incorporation of glucose into teichoic acid in *B. subtilis*, GdaB, has been found to be dependent upon σ^B [1,2]. Moreover, the proposed glycosyltransferase GgaA, involved in the biosynthesis of galactosamine-containing minor teichoic acids in *B. subtilis*, has been shown to belong to the σ^B regulon as well [1,2], indicating a role of the σ^B regulon on cell wall biosynthesis and the surface-specific carbohydrate modifications that are important antigenic determinants assumed to be involved in pathogenesis of bacteria [27]. It is conceivable that the σ^B-dependent SA2219 might have a similar function in *S. aureus*.

SA2298 contains the conserved domain COG4876, which has not been associated with any specific function in bacteria yet. Its *B. subtilis* homologue, YdaT (51% amino acid identity and 65% similarity to SA2298), has been found to belong to the σ^B regulon [1,2]. Although its homologue in *L. monocytogenes* (Lmo0670; 50% amino acid identity and 71% similarity to SA2298) has not been identified to belong to the σ^B regulon yet, its is likely to be σ^B-dependent, as it is translationally coupled with the upstream-located putative oxidoreductase Lmo0669 that has been found to belong to the σ^B regulon [23].

SA0455 is a homologue of the regulatory protein YabJ of *B. subtilis* (58% amino acid identity and 74% similarity) [28,29]. Similar to *B. subtilis*, *sa0455* is preceded by a gene encoding the PurR repressor. YabJ belongs to the highly conserved YjgF family of widely distributed proteins of unclear function [29]. In *B. subtilis*, YabJ stimulates the adenine-mediated repression of the purine biosynthetic

Table 1
Function and genetic organization of the identified *S. aureus* σ^B-dependent genes

Gene name (synonyms)	Operon structure	Function	Coordinates in *S. aureus* N315 genome
sa1984 (*asp23*)	*sa1985* asp23*	alkaline shock protein	AP003136 (163805–163296)
sa1987 (*opuD*)	*opuD sa1986 sa1985 asp23*	glycine betaine transporter	AP003136 (166393–164831)
sa2336 (*clpL*)	*clpL*	ATP-dependent Clp proteinase chain ClpL	AP003137 (221015–223120)
sa0742 (*clfA*)	*clfA*	fibrinogen-binding clumping factor	AP003131 (249484–252453)
sa1452 (*csb8*, *csbD*)	*csb8*	conserved hypothetical protein	AP003134 (157820–157638)
sa1946 (*csb9*)	*csb9*	conserved hypothetical protein	AP003136 (104291–103626)
sa0772	*sa0772*	conserved hypothetical protein, likely paralogue of *csb8*	AP003131 (279504–279698)
sa2219	*sa2219*	predicted membrane protein of the GtrA family	AP003137 (92009–91623)
sa2298	*sa2298*	conserved hypothetical protein	AP003137 (181699–181211)
sa0455	*purR* sa0455*	homologue of *B. subtilis* YabJ, belongs to YjgF family	AP003130 (227948–228328)
sa1143	*glpD* sa1143 miaA*	hypothetical protein, similar to lysophospholipase	AP003133 (105115–106029)
sa0572	*sa0572*	hypothetical protein, similar to esterase/lipase	AP003131 (66307–67095)
sa0359	*sa0359*	hypothetical membrane protein	AP003130 (118691–119263)
sa2309	*sa2309*	conserved hypothetical protein, predicted acetyltransferase	AP003137 (194670–194954)
sa0752	*sa0752*	hypothetical protein	AP003131 (261146–260910)
sa2451	*sa2451*	hypothetical protein	AP003138 (66641–67000)
sa0633	*sa0633*	hypothetical protein	AP003131 (128994–129293)
MW0922	*MW0922*	hypothetical protein	AP003132 (114986–114854)

Asterisks indicate the presence of an internal σ^B-dependent promoter; in all other cases promoters lie to the left of the leftmost gene. Operon structure was predicted based on the translational coupling of the genes directed by the σ^B-dependent promoter.

gene *purA* by the PurR repressor [28]. Although a variety of biological processes have been reported to be influenced by this family (basically implicated in regulation), and crystal structures of two of them (YabJ and YjgF) have been determined, these results have not pointed to a specific biochemical activity of these proteins yet [29].

Both SA1143 and SA0572 are likely involved in lipid metabolism. SA1143 contains the conserved domain COG2267 that is characteristic for lysophospholipases, and SA0572 harbors the domain COG1647 that has been found in some esterases/lipases. Interestingly, the proposed esterase/lipase homologue YvaK of *B. subtilis* was found to belong to the σ^B regulon [1,2].

SA0359 is similar to several hypothetical bacterial membrane proteins. It contains the conserved domain COG3212 of predicted membrane proteins. However, neither its homologue in *L. monocytogenes*, Lmo0047, nor its homologue in *B. subtilis*, YkoJ, has been identified to belong to the σ^B regulons of these strains [1,2,23].

SA2309 contains the domain COG2388 of predicted acetyltransferases. Its homologue in *L. monocytogenes*, Lmo0134, has not been identified to belong to the σ^B regulon of this strain [23]. There is no significant similarity to any protein in *B. subtilis*.

The last four new members of the *S. aureus* σ^B regulon, SA0752, SA2451, SA0633, and MW0922, encode hypothetical proteins with yet unknown functions. They seem to be specific for staphylococci, as no significant similarity with any protein in databases could be detected.

It is worth noticing that almost all the identified σ^B-dependent genes, except *sa1143* and *MW0922*, were recently identified by a DNA microarray-based analysis in three genetically distinct *S. aureus* strains (M. Bischoff, manuscript in preparation), demonstrating the reliability and suitability of the system presented here to identify σ^B promoters. It was surprising that among the 18 identified *S. aureus* σ^B-dependent genes only three (*csb8*, *csb9*, *clpL*) were found previously by a proteomic approach [7]. Therefore, we suppose that this number of σ^B-dependent promoters in *S. aureus* is not the total. A possible explanation why the other σ^B-dependent genes identified by the proteomic approach have not been identified using our two-plasmid system may be that the *S. aureus* library used did not cover the complete genome. Moreover, the *E. coli* two-plasmid system has some limitations. First, it is likely that he promoters requiring transcriptional activation in vivo in *S. aureus* by a specific transcriptional activator cannot be identified by the method. Thus, the system could identify only promoters that are solely recognized by RNA polymerase holoenzyme containing σ^B. The other reason could be that some genes are directed by several close tandem promoters, of which only one is σ^B-dependent and the other one might be dependent upon the housekeeping sigma factor σ^A. The *S. aureus* σ^A-dependent promoters are recognized in *E. coli*, such DNA fragments therefore could not be identified by the two-plasmid system as σ^B-dependent, as they confer *lacZ*α reporter activity also in the absence of induced σ^B.

In conclusion, we have shown that the *E. coli* two-plasmid system with the arabinose-inducible *S. aureus sigB* gene is suitable for the identification of σ^B-dependent promoters and their corresponding genes. Using this system, 18 *S. aureus* σ^B-dependent promoters were identified and the TSPs of the promoters were located. Additionally, the system proved useful for confirming the σ^B dependence of proposed σ^B-dependent promoters. Comparison of the sequences of the identified *S. aureus* σ^B-dependent promoters revealed a consensus sequence (GttTaa-N_{12-15}-gGGTAt) that is highly similar to the consensus sequence of *B. subtilis* σ^B-dependent promoters [17]. Some of the identified *S. aureus* σ^B-dependent genes are supposed to play a role in stress response and virulence.

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